

## AMENDMENTS

### Amendments to the Specification

Please amend the following paragraphs, wherein the deleted matter is shown by strikethrough and the added matter is shown by underlining.

Please delete paragraph [006], and replace it with the following paragraph:

[006] With recent advances in functional genomics and proteomics, there is an urgent need to develop technologies and strategies to detect proteins, multi-protein complexes and protein-protein interactions *in ~~vivo~~ vitro*. For example, there is a lack of novel labeling reagents in performing *in vivo* protein expression and interaction studies. There exist few tools that can be used to visualize protein-protein interaction inside cells in deep tissue, and to track the interaction between drug molecules and the associated protein targets. There is virtually no existing method that enables studies of the dynamics of different intra-cellular molecular processes in deep tissue. This is a daunting challenge in that none of the many approaches under development to address these problems have yet demonstrated compelling promise - even as generally effective laboratory-scale small animal models.

Please delete paragraphs [038] and [041].

Please delete paragraphs [039], [040], and [042], and replace them with the following paragraphs:

[039] Figure ~~12~~ 11 shows results of magnetic resonance imaging of MDBK cells. Figure ~~12A~~ 11A is a graph showing the T2 relaxation times (in ms) of 500 ml cell suspension samples. Column 1 shows values for MDBK cells with MIONs, and Column 2 shows values for the control of MDBK cells alone. Figure ~~12B~~ 11B shows an image of a cross-section of

samples from a 3T Siemens MRI system. The first and fourth samples are control samples of media only; the second sample is a control of MDBK cells with media; and the third sample is MdDBK cells with MIONs and media.

[040] Figure ~~13~~ 12 is a schematic illustration of three different conjugation schemes for linking the delivery peptide to nucleic acid probes. (A) The streptavidin-biotin linkage in which a nucleic acid probe is modified by introducing a biotin-dT to the quencher arm of the stem through a carbon-12 spacer. The biotin-modified peptides are linked to the modified nucleic acid probe through a streptavidin molecule, which has four biotin-binding sites. (B) The thiol-maleimide linkage in which the quencher-arm of the nucleic acid probe stem is modified by adding a thiol group which can react with a maleimide group placed to the C terminus of the peptide to form a direct, stable linkage. (C) The cleavable disulfide bridge in which the peptide is modified by adding a cysteine residue at the C terminus which forms a disulfide bridge with the thiol-modified nucleic acid probe. This disulfide bridge design allows the peptide to be cleaved from the nucleic acid probe by the reducing environment of the cytoplasm.

[042] Figure ~~15~~ 13 shows the detection of Survivin mRNA in live HDF and MiaPaca-2 cells. (A) Strong fluorescence signal was observed in MiaPaca-2 cells after 60 min of incubation with peptide-linked nucleic acid probes. Note that essentially all cells shown fluorescence. (B) Survivin mRNA molecules in MiaPaca-2 cells seemed to be concentrated near one side of the cell nucleus HDF cells. (C) Only very low fluorescence signal can be observed in HDF cells, but with a survivin mRNA localization pattern.

Please delete paragraph [048], and replace it with the following paragraph:

[048] In certain preferred embodiments, the surface of the biocompatibly coated magnetic nanoparticles of the present invention comprise a delivery ligand. As used herein, a “delivery ligand” refers to a molecule that is capable of effectively delivering the magnetic nanoparticle probe across a cell membrane or across both a cell membrane and additional intracellular

organelle membrane. In a further preferred embodiment for intracellular delivery, the delivery ligand selected allows for either endocytic or non-endocytic uptake pathways. In a more preferred embodiment, the magnetic nanoparticle probe is delivered using a non-endocytic uptake pathway, using a delivery ligand selected from the group consisting of a protein transduction domain peptide (PTD) and a cell penetrating peptide (CPP). In a further preferred embodiment, the delivery ligand is a TAT peptide. In other embodiments, the delivery ligand may be selected from a group consisting of HIV-1 TAT, HSV VP22, ANTP, poly-Arginine, and Arginine-rich peptides such as W/R, NLS\*, AlkCWK<sub>18</sub>, DiCWK<sub>18</sub>, transportan, DipaLytic, K<sub>16</sub>RGD, P1, P2, P3, P3a, P9.3, Plae, Kplae, cKplae, MGP, HA2, LARL46, Hel-11-7, KK, KWK, RWR, and Loligomer. The specific sequences of certain delivery peptides are disclosed in Schwartz and Zhang (2000), and are as follows:

TAT (with amino acid sequence YGRKKRRQRRR) (SEQ ID NO:1)

HSV VP22 DAATATGRSAASRPTERPRAPARSASRPRRPVE (SEQ ID NO:2)

ANTP (RQIKIWFQNRRMKWKK) (SEQ ID NO:3)

W/R (RRWRRWWRRWWRRWRR) (SEQ ID NO:4)

NLS (TPPKKKRKVEDP) (SEQ ID NO:5)

AlkCWK<sub>18</sub> (CWKKKKKKKKKKKKKKKKKKKK) (SEQ ID NO:6)

DiCWK<sub>18</sub> (K<sub>18</sub>WCCWK<sub>18</sub>) (SEQ ID NO:7)

Transportan (GWTLNSAGYLLGKINLKALAALAKKIL) (SEQ ID NO:8)

DipaLytic (GLFEALEELWEAK) (SEQ ID NO:9)

K<sub>16</sub>RGD (K<sub>16</sub>GGCRGDMFGCAK<sub>16</sub>RGD) (SEQ ID NO:10)

P1 (K<sub>16</sub>GGCMFGCGG) (SEQ ID NO:11)

P2 (K<sub>16</sub>ΘICRRARGDNPDDRCT) (SEQ ID NO:12)

P3 (KKWKMRNRFQWVKVQRbAK (B) bA) (SEQ ID NO:13)

P3a (VAYISRGGVSTYYSDTVKGRFTRQKYNKRA) (SEQ ID NO:14)

P9.3 (IGRIDPANGKTKYAPKFQDKATRSNYYGNSPS) (SEQ ID NO:15)

Plae (PLAEIDGIELTY) (SEQ ID NO:16)

Kplae (K<sub>16</sub>GGPLAEIDGIELGA) (SEQ ID NO:17)  
cKplae (K<sub>16</sub>GGPLAEIDGIELCA) (SEQ ID NO:18)  
MGP (GALFLGFLGGAAGSTMGAWSQPKSKRKV) (SEQ ID NO:19)  
HA2 (WEAK (LAKA)<sub>2</sub>LAKH(LAKA)<sub>2</sub>LKAC) (SEQ ID NO:20)  
LARL<sub>4</sub> ((LARL)<sub>6</sub>-NH-CH<sub>3</sub>) (SEQ ID NO:21)  
Hel-11-7 (KLLKLLKLWLKLLKLL) (SEQ ID NO:22)  
KK ((KKKK)<sub>2</sub> GGC) (SEQ ID NO:23)  
KWK ((KWKK)<sub>2</sub> GCC) (SEQ ID NO:24)  
RWR ((RWRR)<sub>2</sub> GGC) (SEQ ID NO:25)  
Loligomer ((K9K2) (K4) (K8) GGKKKKK-NLS) (SEQ ID NO:26) wherein NLS consists of the NLS sequence disclosed in this paragraph as SEQ ID NO:5.

Immediately before paragraph [094], please add the following heading:

#### EXAMPLE 1

Please delete paragraphs [0104] and [0105], and replace them with the following paragraphs:

[0104] The mMIONs with both TxRed and TAT peptide attached were successfully delivered into human dermal fibroblast (HDF) cells and kidney-derived MDBK cells (data not shown), ~~as shown respectively in Figures 11A and 11B.~~ HDF cells were plated on 8-well Nalgene Nunc cell culture plate and allowed to grow for 48 hours. A 1:4 dilution of the original mMIONs-Tx Red-peptide complex in FBM2 media formulation provided by Cambrex (New Jersey) was added to cells and incubated for an hour. Cells were washed in PBS buffer two times and imaged using a Zeiss confocal microscope (~~Figure 11~~ data not shown). MDBK cells were treated similarly.

[0105] To further validate the delivery of the micelle-MIONs into live MDBK cells, T<sub>2</sub> relaxation times were measured and MRI contrast images were obtained. For T<sub>2</sub>

measurements, a Bruker Minispec Analyzer MQ20 was used. Cells were grown in a T25 culture plate (approximately  $10^6$  cells) and then incubated with a 1:4 dilution of mMIONs and media (as above). After 1 hour of incubation, cells were washed two times with PBS buffer and trypsinized to remove the cells from the plate. Cells were resuspended in a final volume of 1.5 ml of media. 500 ul of cell suspension was placed in a 10 mm sample tube for  $T_2$  measurements.  $T_2$  values were determined using the cpmb sequence.  $T_2$  relaxation time determined for cells with mMIONs was  $623 \pm 2$  ms;  $T_2$  relaxation time for cells without mMIONS was  $1503 \pm 20$  ms (See Figure 12(a) 11(a)). Controls for this experiment were MDBK cells without mMION incubation. This cell sample was then imaged on a 3T Siemens MRI system, and good contrast was obtained for the cells that contained micelle-MIONs (Figure 12(b) 11(b)).

Please delete paragraph [112], and replace it with the following paragraph:

[112] To evaluate the difference between the MRI signal generated by the hybridized complex as compared to the individual oligonucleotide samples  $T_2$  relaxation time was measured for the same samples used for the optical detection using FRET. In this assay, the total MION particle concentration in the Cy3-MION + Cy5-MION sample was the average of the MION concentration in the individual samples (Cy3-MION, Cy5-MION). Even though the samples initially had the same concentration of MION particles for conjugation of the oligonucleotides, there are small variations in concentration of the two samples (Cy3-MION, Cy5-MION). The MRI results are shown in ~~Table 2~~ Table 1. The results clearly indicate a shift in the  $T_2$  relaxation time (ms) of the Cy3-MION + Cy5-MION as compared to the Cy3-MION and Cy5-MION samples.

Please delete paragraphs [0115] to [0117], and replace them with the following paragraphs:

[0115] The first model involves a viral infection (influenza virus A) in rats. Virus is injected into one of animal's hind leg and allowed to develop for 24 hours before imaging. The nanoprobe, designed to target the eotaxin mRNA,<sup>46</sup> mRNA, are then be injected. The imaging studies will compare the two hind legs and also assess the heterogeneity of the infected leg. Contrast will be quantitatively assessed by comparing image intensity of the infected leg with the control leg.

[0116] The second model is a brain injury model of rats induced by inertial acceleration. It has been shown that the mRNA expression of cytochrome c oxidase II (COII), a mitochondrial encoded subunit of complex IV, is upregulated following traumatic brain injury (TBI).<sup>47</sup> (TBI). The severity of the injury will be made very low so that no edema will be present and little contrast is seen on conventional MRI. The animal will be imaged before the injury, after the injury but without the nanoprobe, and after the injury with nanoprobe. Contrast to noise ratios between the injured area and the surrounding tissue will be calculated.

[0117] Previous in vivo studies in the liver demonstrated robust contrast at a spatial resolution of ~~1 mm~~.<sup>48</sup> 1 mm. That study was performed at 0.47 T and using a human head coil. For the proposed studies, a specialized coil will be used that is much smaller than the head coil used in the liver study. The studies will be performed at 3 T. With the increase in field strength and coil sensitivity, which will increase the signal-to-noise ratio and the relaxation effect of the magnetic nanoparticles, we expect a substantial increase in detection sensitivity, allowing us to achieve a resolution of 0.1 mm in mice. This resolution should be sufficient for studying infected areas with sufficient spatial resolution.

Please delete paragraphs [0120] to [0122], and replace them with the following paragraphs:

[0120] To demonstrate the rapid and sensitive detection of mRNA in living cells, we developed peptide-linked nucleic acid probes that possess self-delivery, targeting and reporting functions. We conjugated the TAT peptide to nucleic acid probes using three

different linkages and demonstrated that, at relatively low ( ~~$\geq 200$  nM~~) ( $\geq 200$  nM) concentrations, these nucleic acid probe constructs were internalized into living cells within 30 ~~min~~ minutes with nearly 100% efficiency. Further, peptide-based delivery did not interfere with either specific targeting by or hybridization-induced fluorescence of the probes. We could therefore detect human GAPDH and Survivin mRNAs in living cells fluorescently, revealing intriguing intracellular localization patterns of mRNA.

[0121] We designed and synthesized peptide-linked nucleic acid probes targeting the human GAPDH (glyceraldehyde 3-phosphate dehydrogenase) and survivin mRNAs, as well as nucleic acid probes with a 'random' probe sequence. The specific design of these nucleic acid probes, and the sequence of the 11 amino-acid TAT-1 peptide used in the study are shown in ~~Table 3~~ Table 2. The GAPDH beacon is comprised of a 19-base probe domain targeting the Exon 6 region of GAPDH gene flanked by complementary 5-base sequences that hybridize to form the stem. The survivin beacon has a 16-base target sequence with a similar design of the stem. The 'random' beacon was designed as a negative control, with a 17-base probe sequence that does not have any match in the entire human genome.

[0122] Three conjugation strategies were developed in attaching the delivery peptide to nucleic acid probes, as illustrated in ~~Figure 13~~ Figure 12. In the first approach (Figure ~~13A~~ 12A), peptides were linked to a nucleic acid probe through a streptavidin-biotin bridge by introducing a modified oligonucleotide, biotin-dT, to the quencher arm of the stem through a carbon-12 spacer. The peptide-linked nucleic acid probe consisted the biotin-modified nucleic acid probe, a streptavidin molecule, and biotin-modified TAT-1 peptides. Since each streptavidin molecule has four biotin-binding sites, we were able to link biotin-modified nucleic acid probes and delivery peptides on the same streptavidin molecule. The stoichiometry was controlled so that the probability of having more than one nucleic acid probes linked to the same streptavidin is small. In the second design (Figure ~~13B~~ 12B), we placed a thiol group to the quencher-arm of the nucleic acid probe stem through a carbon linker; the thiol group then reacted with a maleimide group added to the C terminus of the

peptide to form a thiol-maleimide linkage (Figure ~~13B~~ 12B). Both the streptavidin-biotin bridge and the thiol-maleimide linkage are stable in the cell cytoplasm. As the third approach, we functionalized the TAT-1 peptide by adding a cysteine residue at the C terminus which forms a disulfide bridge with the thiol-modified nucleic acid probe as shown in Figure ~~13C~~ 12C. This cleavable design was based on the rationale that the reducing environment of the cytoplasm will cleave the disulfide bond once the construct enters the cell, thereby separating peptide from probe.

Please delete paragraphs [0124] to [0126], and replace them with the following paragraphs:

[0124] To demonstrate the self-delivery and mRNA targeting functions of peptide-linked nucleic acid probes, we first detected mRNA of a housekeeping gene human GAPDH in normal human dermal fibroblast (HDF) cells. After just 30 ~~min~~ minutes of incubation with TAT-peptide conjugated GAPDH-targeting nucleic acid probes, we observed clear and localized fluorescence signal in HDF cells as a result of nucleic acid probe-target mRNA hybridization for all three conjugation schemes, i.e., thiol-maleimide (~~Figure 14A~~), disulfide bridge (~~Figure 14B~~) and streptavidin-biotin (~~Figure 14C~~) (data not shown). In contrast, peptide-linked random-sequence nucleic acid probes with streptavidin-biotin conjugation gave essentially no signal 30 minutes after delivery (data not shown) (~~Figure 14D~~). Similar results were obtained using random-sequence nucleic acid probes with thiol-maleimide and disulfide linkages for peptide (data not shown). This demonstrates that peptide-linked nucleic acid probes remained highly specific in living cells after internalization. Further, we found that GAPDH mRNAs displayed a very intriguing filament-like localization pattern in HDF cells, with a clear tendency of surrounding the cell nucleus and following the cell morphology (data not shown) (~~Figures 14A-C~~). Interestingly, nucleic acid probes with the cleavable (thiol-cysteine disulfide bridge) design seemed to give better localization patterns than those with the thiol-maleimide linkage, and the latter seemed to perform better than nucleic acid probes



with the streptavidin-biotin linkage. Cleavage of the delivery peptide from the construct may have provided nucleic acid probes a better access to target mRNA molecules, although more studies of this phenomenon are required to validate this assumption. It is likely that a nucleic acid probe with a relatively bulky streptavidin molecule is less able to penetrate into the secondary structure of the GAPDH mRNA, thus reducing its ability to seek out its targets. Almost all the HDF cells exposed to GAPDH peptide-linked nucleic acid probes showed strong fluorescence signal, implying a near 100% delivery efficiency (data not shown).

[0125] Similar results were obtained after 60 minutes of incubation (data not shown ~~Figures 14E-H~~). About the same level of fluorescence was observed for GAPDH-targeting nucleic acid probes with different linkages for peptide, whereas the random-sequence nucleic acid probes did not give much signal. Even after 90 minutes, there was essentially no increase in the signal level (data not shown), indicating that most of the peptide-linked nucleic acid probes entered the HDF cells within the first 30 minutes. Further, fluorescence signal levels and mRNA localization patterns in HDF cells were similar for experiments at three different nominal nucleic acid probe concentrations (0.25  $\mu$ M, 0.5  $\mu$ M and 1.0  $\mu$ M). Due to the peptide conjugation process, it was estimated that, with 0.25  $\mu$ M nominal nucleic acid probe concentration, the actual concentration of peptide-linked nucleic acid probes used in the assay was about 150–200 nM.

[0126] To demonstrate the ability of nucleic acid probes to determine gene transcription levels, we observed expression of Survivin mRNA in live HDF and MiaPaca-2 cells. We and others have demonstrated that the Survivin expression level is very low in HDF cells, whereas in MiaPaCa-2 cells the level is relatively high. After 60 minutes of incubation with peptide-linked nucleic acid probes, the fluorescence signal in MiaPaca-2 cells was quite high, as shown in Figure ~~15A~~ 13A, but in HDF cells, only very low fluorescence signal can be observed (Figure ~~15C~~ 13C). In addition, Survivin mRNAs shown an intriguing localization pattern, i.e., the Survivin mRNA molecules in MiaPaCa-2 cells seemed to be concentrated near one side of the cell nucleus (Figure ~~15B~~ 13B). Although with very low

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expression level, Survivin mRNA localized in HDF in a similar fashion (Figure ~~15C~~ 13C). Previous research suggested that the expression level and localization of Survivin may be an important indicator for cancer progression or prognosis.